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Ocular Oncology Update

Animal models in retinoblastoma research

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Abstract

Advances in animal models of retinoblastoma have accelerated research in this field, aiding in understanding tumor progression and assessing therapeutic modalities. The distinct pattern of mutations and specific location of this unique intraocular tumor have paved the way for two types of models- those based on genetic mutations, and xenograft models. Retinoblastoma gene knockouts with an additional loss of p107, p130, p53 and using promoters of *Nestin*, *Chx10*, and *Pax6* genes show histological phenotypic changes close to the human form of retinoblastoma. Conditional knockout in specific layers of the developing retina has thrown light on the origin of this tumor. The use of xenograft models has overcome the obstacle of time delay in the presentation of symptoms, which remains a crucial drawback of genetic models. With the advances in molecular and imaging technologies, the current research aims to develop models that mimic all the features of retinoblastoma inclusive of its initiation, progression and metastasis. The combination of genetic and xenograft models in retinoblastoma research has and will help to pave way for better understanding of retinoblastoma tumor biology and also in designing and testing effective diagnostic and treatment modalities.

Keywords: Retinoblastoma, Knock-out genetic model, Xenograft models, Preclinical models

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Introduction to animal models in cancer research

Animal models are an integral part of preclinical research in the field of oncology. Non-human tumor models have helped to identify the course of tumorigenesis and evaluation of diagnostic and therapeutic protocols in several human cancers such as colon, breast, ovarian, and hepatocellular carcinomas, and ocular melanomas.[1–6] In vitro studies have their own limitations of being conducted devoid of the complex microenvironment that exists within the human body. Interrogation of the cellular mechanisms of tumor progression within the complexity of an organism can help expose the full extent of pathophysiological changes that take place in neoplasms.

Retinoblastoma

Retinoblastoma is the most common pediatric ocular malignant tumor occurring in 1 of every 15,000–20,000 live births.[7,8] This tumor is caused due to inactivation of both the alleles of the Retinoblastoma (Rb) gene resulting in the defective formation of pRB protein. pRB is a major tumor suppressor gene that is involved in cell cycle progression, terminal differentiation and DNA replication.[9] Loss of pRB activity in the retinal progenitor cells leads to impaired cell cycle and uncontrolled cell proliferation. Retinoblastoma manifests in both unilateral and bilateral forms depending on whether it is sporadic or familial.[10] The understanding of genetic inheritance and advances in diagnostic techniques have not only led to early diagnosis and genetic prediction but have paved way for the first of its kind successful preimplantation genetic diagnosis. Xu et al. reported that it was possible to screen embryos with RB1 mutations, implant a healthy embryo following in vitro fertilization and subsequently achieve a healthy pregnancy and delivery.[11] Also, accurate identification of RB1 mutation enables early diagnosis and management of family members at risk for developing retinoblastoma.[12]

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Retinoblastoma is currently considered highly treatable, with an overall 3-year survival rate of over 90%.[13] However, it is invariably fatal when left untreated. Prior to the 1990's, the standard treatment approach to unilateral RB was enucleation and in the bilateral cases, the treatment typically involved enucleation of the worst eye and external beam radiation of the other eye. Systemic chemotherapy is now the treatment of choice for retinoblastoma with an effort to salvage life, eye, and vision. However, it has been observed that most of the retinoblastoma cases show continued cellular activity even in eyes treated with primary systemic chemotherapy regimen.[14] The prognosis of the disease is affected by the time of diagnosis and tumor stage.

Genetics of retinoblastoma

The predisposition to retinoblastoma was predicted by Alfred Knudson following statistical analysis of occurrence in the early 1970's. Identification of retinoblastoma gene in 1987 confirmed his hypothesis. The bilateral form is hereditary while the unilateral form is generally non-hereditary.[15] In the hereditary form, the predisposition to tumor formation is inherited from a parent who is a carrier of one mutant allele of the RB1 gene. The presence of one copy of the mutant gene through germ line transmission predisposes the child to the loss of second copy at a rate 1000 times more likely than a spontaneous mutation. This form is more likely to be multifocal since a copy of mutant RB1 is present in all cells and mutation of the second allele could occur in several retinal cells. Lack of RB1 in non-retinal cells can also predispose patients to second malignant neoplasm like osteosarcomas. Unilateral retinoblastoma involves somatic mutation/loss of both copies of the RB1 gene in the developing retina and is generally unifocal.

Tumorigenesis is a multiple step process with a series of mutations that render cells capable of indefinite proliferation, resistance to cell death, inducing angiogenesis, evading growth suppressors, activation of metastasis, evading immune destruction and deregulating cellular energetics. These neoplastic cells have high predilection toward genomic instability and triggering inflammatory response.[16] In Retinoblastoma, the loss of function of the RB1 gene initiates retinoma leading to genomic instability.[17] Retinomas with non-proliferative areas have extra copies of genes on chromosome 1q, that includes KIF14 and MDM4. Retinoblastomas with increased mitotic activity have multiple copies of oncogenes such as KIF14, E2F3, DEK and MYCN; in conjunction to loss of CDH11, a tumor suppressor gene.[18,19] The complete sequence of events promoting retinoblastoma tumorigenesis is still unknown and techniques such as whole-genome sequencing of cancers would help unravel the transformation of benign retinoma to malignant retinoblastoma.[20]

The need for animal models in retinoblastoma research

Retinoblastoma cases that warrant enucleation are presented in an advanced stage and it has been practically impossible to study the origin of the tumor in human samples owing to its late presentation and lack of enough viable tissues. Hence, animal models are indispensable tools to help study retinoblastoma origin and tumorigenesis.[21,22] Spontaneous generation of this tumor seems to be limited only to humans and several challenges lie in creating an animal model that mimics retinoblastoma structurally and functionally.

Transgenic models of retinoblastoma

During the past three decades several genetic murine models of retinoblastoma have been developed with moderate to high similarity with human form of the tumor. With the identification of the role of p107 in mouse retina, the knockout of both the genes was necessary to generate retinoblastoma in murine eyes.[21,23]

The retinoblastoma transgenic animal models are detailed below:

LH-beta T-Ag models

This is one of the first and widely studied transgenic models that was developed in 1990 by Windle JJ et al. This model expresses the oncogenic SV40 early region under the control of luteinising hormone β sub-unit (LHβ) promoter in the gonadotrope cells of the anterior pituitary region.[24] The oncogenes present in both Large-T and small-t of the SV40 early region were hypothesized to cause transformation as these oncoproteins bind to the pRB family, p53 and phosphatase pp2A.[25] Following this model, retinoblastoma has also been developed using mice expressing T-Ag/t-Ag from the IRBP promoter.[26,27] These models showed neuronal characteristics of human retinoblastoma histologically but the question of cell of origin still remained unanswered until the last decade. Both Flexner-Wintersteiner and Homer-Wright rosettes were observed by light and electron microscopy by five months of age. However, Pajovic et al. have recently studied T-Ag protein expression in the retina to track tumor development from the earliest stages.[28] They reported that Tag expression starts by P8 in the nuclei within the inner nuclear layer of the developing retina and then an increase in number by P21 following which there is a decline until P28. This decline of TAg expression was coupled with increase in expression of activated Caspase-3 indicating apoptosis. Expression of Müller glial markers in these Tag positive cells was observed at P8 and P9 with the absence of amacrine, horizontal and bipolar cells. This study concluded that the cell of origin in these tumors belongs to a sub group of progenitor like Muller glial cells that undergoes transformation following Tag expression. Wadhwa et al. developed a Pax6 driven T-Ag model and established a Rb murine cell line using the tumors from P7 mice.[29] Upon characterization, they have identified the presence of a subpopulation of tumor initiating cells that express CD133, Nestin and Sox2. The CD133+ cells were observed to be capable of generating neurospheres in vitro and form transplantable tumors in vivo identical to their parent tumor.

The T-Ag model has been widely used in evaluating therapeutics such as local carboplatin therapy, radiation therapy, cryotherapy, and vascular targeting therapies.[30–32] Jockovich et al. showed that conventional therapies (local carboplatin chemotherapy and external beam radiotherapy (EBRT)) and vascular targeting chemicals, such as anecortave acetate, increase apoptotic cell death, while not having a significant effect on necrosis in this murine model of retinoblastoma.[32]

Timothy et al. observed that in 5-week transgenic BLH SV-40 T-antigen-positive mice with retinoblastoma, subconjunctival delivery of carboplatin in increasing doses effectively inhibits intraocular tumor growth in a dose-dependent fashion. The group also observed that cryotherapy does not increase tumor control in this genetic retinoblastoma model.[31] Intravitreal carboplatin in combination with EBRT also showed better tumor growth control in these mice.[30]

Assessing tumor growth in vivo has been a challenge but studies conducted on these transgenic mice using Optical coherence tomography (OCT) has allowed the quantification of tumor growth from the earliest stages of tumor initiation to advanced tumors.[33,34]

These models have been invaluable in conducting preclinical studies for developing better therapeutics but one of the main disadvantages was the use of viral oncoproteins in inducing the tumor, the functions and interactions of which are still not fully elucidated. This model also showed lack of focal and clonal tumors characteristic of human retinoblastoma. These limitations warranted the development of conditional knockout models that not only mimicked the histological features of the human tumor but also its tumorigenesis.

Retinoblastoma knockout models

With the advent of gene knock-out technology, several groups attempted to generate Rb mouse models by creating chimeric animals in which the retina alone was constituted of Rb−/−cells. But these chimeras did not develop retinoblastoma in spite of the Rb−/− cells contributing to the developing central nervous system.[35,36] It was however observed that the developing retina of chimeric Rb−/− embryos exhibited ectopic mitoses and extensive cellular degeneration. The role of another protein, p107, was then identified by Robanus-Maandag et al. to be involved in preventing retinoblastoma in mice lacking a functional Rb gene.[23] The protein, p107, shares strong similarities with the RB1 in terms of its sequence and biochemical parameters.[37] It was also reported that in postnatal murine retinas and their explant cultures lacking Rb, p107 gene was upregulated, unlike human retina, which supported the hypothesis that p107 compensates for Rb.[38,39]

The developmental lethality of Rb−/−;p107−/− was found to be very high and hence in an attempt to generate breedable Rb mutant mice, Cre-Lox technology was used to selectively knock out the genes in the developing retina.[40] This study showed that loss of Rb, p107, p53 in photoreceptor cells did not lead to any phenotypic changes toward retinoblastoma development. This study paved way for the use of Cre transgenic technology to create breedable Rb knockout models with the help of Nestin, Chx10 and Pax-6 promoters in retinal progenitor and other cells.[38,41–43] These models take 3–9 months to develop detectable tumor and show positive staining for progenitor, amacrine and Müller glial cells. Pax-Cre and Nestin-Cre were unsuccessful preclinical models due to their late onset, low penetrance and cell non-autonomous effects and could not be used for further studies. Rb deletion models with chimera systems and different Cre-transgenic lines have many similarities in their phenotypes such as mitotic figures, high levels of cell death in inner retina from E16.5–18.5,[36] ectopic proliferation in retinal ganglion cell layer,[44] photoreceptor degeneration[23,38]; with the most prominent effect being the extension of proliferative period of retinogenesis.[38,42,43] It was also observed that upon Rb deletion, high levels of apoptosis can occur in specific cell types. For instance, in α-Cre Rblox/lox mice, the ganglion, bipolar as well as a large majority of rod cells were completely degenerated.[42] This defect was not observed in studies that used Chx10Cre Rblox/lox retinas, which did not exhibit bipolar cell death.[45] The diverse phenotypic differences between the models could be explained by the heterogeneity in the genetic background across different strains and the timing of Cre expression across the cells types.[21]

The inclusion of an additional mutation of p107 in these models resulted in the enhancement of several development phenotypes seen with retinal Rb loss. This revealed that there is a functional synergy between these family members.[23,38,42] A rise in the embryonic retinal proliferation and apoptosis was observed in Rb−/−;p107−/− models. Many of the amacrine, horizontal and Muller cells survived Rb and p107 mutation and a group of these animals developed retinoblastoma. A noteworthy point from these models was that since retinoblastoma arose from highly apoptosis-sensitive cells, the cell of origin of these tumors bore inherent resistance to cell death. This phenomenon is quite similar to human retinoblastomas which also display a high rate of apoptosis. Markers for amacrine, horizontal and glial cells have been reported in retinoblastomas lacking Rb and p107.[23,43,46,47] The major setbacks of this model were incomplete penetrance and delayed tumorigenesis which led to the generation of Rb/p130 DKO models.

The role of p130 in retinal development, cell cycle exit and tumor suppression was elucidated in α-Cre Rblox/lox p130−/−, NesCre1 Rblox/lox p130−/− and in Rb−/−p130−/− chimeras.[38,43,48] Histological defects in the embryonic retinal development observed in the NesCre1 Rb/p107 DKO retinas were absent in these models [38]. Chimeric animals lacking Rb and p130, NesCre1 Rblox/lox p130−/− and α-Cre Rblox/lox p130−/− mice all form retinoblastomas with heterogeneous marker expression including horizontal and amacrine markers.[38,43,48] α-Cre Rblox/lox p130-/-animals showed lesions resembling retinoblastoma, consistent with Homer-Wright rosettes, that were observed as early as PND21 to PND31 at the extreme periphery of the retina.[43] These tumors then grow to fill the vitreous cavity and a portion of the growing cells extend into the brain by invading the optic nerve. These findings are similar to cases with advanced retinoblastoma metastasizing to the brain. The α-Cre Rb / p130 DKO mouse is a pertinent model for studying advanced retinoblastoma given the rapid tumor progression and metastasis. Rb/p130 DKO retinoblastomas appear similar to Rb/p107 DKO retinoblastomas upon histological examination, and both resemble human retinoblastomas with neuroblastic differentiation. To further elucidate the role of Rb family members-Rb1, p107 and p130; Ajioka et al. generated mice that express a single copy of each in the developing retina.[49] They observed that in p107(+/−) retina, the horizontal neurons initially differentiate normally but after several weeks, they re-enter the cell cycle, clonally expand and form bilateral retinoblastoma that metastasized into the bone marrow. The horizontal cells were not affected in the other knock-out mice (Rb(+/−);p107(−/−);p130(−/−) or Rb(−/−);p107(−/−);p130(+/−)), thereby indicating that a single copy of Rb or p130 controlled horizontal cell proliferation and prevented its clonal expansion. This study reported for the first time that differentiated neurons were capable of re-entering the cell

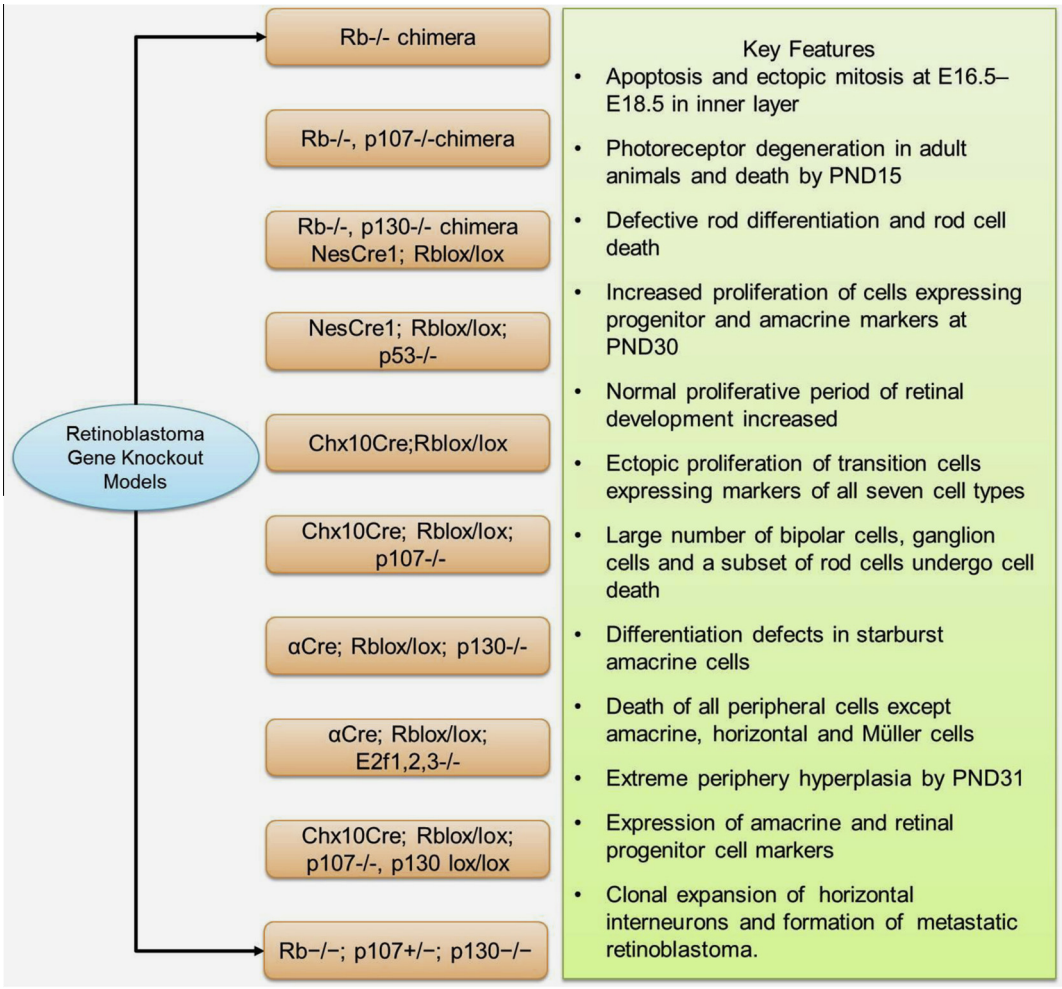

Figure 1. Genetic Models of Retinoblastoma with their key features.

cycle and forming invasive tumors suggesting that retinoblastoma arises from dedifferentiation of horizontal cells.[49] An extensive study was done recently by McEvoy J et al. on six mouse lines that develop retinoblastoma using the Chx10-Cre system.[50] The knockout mice were found to be similar to each other and to human retinoblastoma in their molecular profiles. The cell type signatures, neuroanatomic, neurochemical and morphometric features most common to human and mice models belonged to the amacrine cell differentiation. The summary of established Retinoblastoma knock-out models along with some of their salient features are illustrated in Fig. 1.

The transgenic models have also been used extensively in assessing existing and novel therapeutic modalities. Laurie et al. showed that in a genetic model generated by injecting a replication-incompetent retrovirus encoding the E1A 13S oncogene into newborn p53-deficient mice, which induced clonal retinal tumors arising from one to five individual foci; the combination of topotecan with carboplatin would be a potent alternative to the current triple drug therapy (carboplatin/etoposide/vincristine).[51]

Xenograft models of retinoblastoma

The development of Rb xenograft models has facilitated rapid in vivo assessment of cell lines and tumor tissues in immunocompromised animals. One of the early studies was done in immunosuppressed rabbits by McFall et al. where an inoculum of 10^7 cells from Rb cell lines, Y79 and WERI-Rb, was injected in the anterior chamber of the eye which resulted in tumor growth.[52] The tumors were found to exhibit maximum growth as observed by slit-lamp between day 17–22 post injection. Histological sections confirmed the presence of tumor cells along with corneal vascularization and edema. Kang et al. developed a rabbit model of retinoblastoma using 10^6 WERI-Rb cells injected in the sub-retinal space.[53] They reported tumor, growth detectable with fundoscopy, starting from week 1 post injection with vascularization starting from week 5 and continued tumor growth up to week 8. The histological sections confirmed the intraocular tumor in the sub-retinal space near the optic nerve and vitreous cavity lesions with lack of metastatic phenotype. Blood vessels surrounded by viable tumor cells and necrotic areas similar to that of human retinoblastoma were also observed.[53] The major limitation of this model was that the tumors grow from the sub-retinal space and not from the retina.

Furthermore, tumor growth was achieved in athymic nude mice when primary cells and Rb cell line-Y79 and WERI were injected intraocularly which were reproducible.[54,55] The primary tumors that grew within the eyes remained histologically similar to the original tumors. These tumors could be

serially transplanted into subsequent athymic nude mice and similar growth patterns were achieved. Chévez-Barrios et al. confirmed that Y79 cells represented metastatic phenotype showing invasion into the retina, subretinal space, choroid, optic nerve head, and anterior chamber of the eye, progressing into the subarachnoid space and focally invading the brain. On the contrary, WERI cells produced tumors that were localized in the eye with only anterior choroidal invasion at late stages.[56] Cell number ranging from 10^3 to 10^7 injected intraocularly (intravitreal, anterior chamber, and subretinal) showed positive tumor growth in several xenograft models of retinoblastoma. These models are widely used for testing the efficacy of existing and new chemotherapeutic agents and photodynamic therapy.[51,57,58]

Advances in non-invasive methods of studying in vivo tumor growth and distant metastasis have been achieved using xenograft models of retinoblastoma. Some of them include microcomputed tomography (microCT), magnetic resonance imaging (MRI), positron emission tomography, fluorescence imaging and bioluminescence imaging (BLI) .Xunda Ji et al. showed that using BLI it was possible to monitor intraocular tumor growth and metastasis in vivo. In their study, Y79-GFP-luc cells and HXO-Rb44-GFP-luc cells were used to induce tumors in nude mice. All the unilateral sub-retinal injected mice developed retinoblastoma which did not exhibit metastasis. However, in one of the bilateral subretinal injected mice, brain metastasis was observed 10 weeks post initial detection of luciferase activity.[59] Since these tumors failed to produce distant metastasis, the authors injected the labeled cells directly into the systemic circulation. They noted that a large number of the mice receiving intracardiac injection formed systemic metastatic disease (9/15) whereas of those mice receiving tail vein injection, only 1 mouse (of 16) developed metastasis. The sites for metastasis were most commonly observed to the lymphatic system (47%), bone (40%), and brain (13%).[59]

The major setback to using xenograft models is that the tumor microenvironment is altered when human cells are injected into the rodent/rabbit eyes. This could possibly lead to differences in the genetic heterogeneity and derangement of normal tumor niche. Therefore, these models should be considered with caution and as an intermediate between in vitro cell culture and retinoblastoma genetic models.

Summary and future directions

Retinoblastoma research has progressed rapidly in the last three decades owing to the improvement in developing animal models that closely resemble the human malignancy. Insights into the role of tumor suppression by the Rb gene have also helped in understanding the generation of Rb tumors and its course of tumorigenesis. An ideal Retinoblastoma animal model should exhibit all the features of the human form that include initiation at the cell of origin, clonal expansion, intraocular spread (endophytic/exophytic), and exophytic spread (contiguous through optic nerve and hematogenous spread. Generation of these animal models would pave way for developing powerful diagnostic tools by marker studies and also in testing the efficacy of various treatment modalities and predicting prognostic markers.

Conflict of interest

The authors declared that there is no conflict of interest.

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